LOW MOLECULAR WEIGHT PROTEINS IN SECONDARY LYSOSOMES AS ACTIVATORS OF DIFFERENT SPHINGOLIPID HYDROLASES

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1. Introduction

Various lysosomal sphingolipid hydrolases have been highly purified and characterized with synthetic substrate analogues, e.g., *p*-nitrophenyl derivatives. During purification these lysosomal enzymes lose their activity towards their physiological substrates, e.g. [1,2]. This activity can, however, be restored in vitro by the addition of either ionic or nonionic detergents, such as bile acids, for review see [3,4], or by a low molecular weight physiological activator protein [5–12].

Recently, we demonstrated that the activator protein of cerebroside sulphatase was lysosomal in origin [13]. In this paper we report the isolation of a low- and a high mol. wt. protein fraction from highly purified secondary lysosomes, prepared after loading with Triton WR-1339 or iron. The low molecular weight fraction, which was heterogeneous as revealed by polyacrylamide gel electrophoresis, was itself enzymically inactive, but stimulated sphingolipid hydrolase activity associated with the high mol. wt. fraction when tested with physiological substrates. In addition to its effect on cerebroside sulphatase (EC 3.1.6.8.) [13], this fraction also stimulated a number of other sphingolipid hydrolases, e.g., cerebroside galactosidase (EC 3.2.1.46.), cerebroside glucosidase (EC 3.2.1.45.), β-*N*-acetylgalactosaminidase (EC 3.2.1.52.) as well as sphingomyelinase (EC 3.1.4.12.), associated with the high mol. wt. fraction. These observations clearly indicate that the enzymic degradation of sphingolipids in the lysosomes depends upon the presence of low mol. wt. activator proteins, thus suggesting a special mechanism of sphingolipid hydrolysis within these organelles.

2. Materials and methods

2.1. *Isolation of secondary lysosomes*

Secondary, i.e. Triton WR-1339- and iron-loaded, lysosomes, were prepared according to described procedures [14–17] with minor modifications [13].

2.2. *Isolation of the activator protein fraction*

The activator protein fraction was isolated from secondary lysosomes as described in detail elsewhere [13]. The procedure involved the disruption of the lysosomes by osmotic shock followed by high speed centrifugation, polyacrylamide gel electrophoresis of the lysosol (cf. fig.1), extraction of the low mol. wt., enzyme free, protein fraction from the gel, adsorption onto DEAE ion-exchange cellulose (Whatman DE-52), desalting of the protein eluate by gel filtration (Sephadex G-10) and finally concentration of the protein fraction by freeze-drying.

2.3. *Isolation of acid hydrolases*

A high mol. wt. protein fraction, devoid of activator protein and containing the acid hydrolases, was isolated by a procedure similar to that described above: extraction of the protein fraction from the same polyacrylamide gel (cf. fig.1) used for the preparation of the activator protein, separation of the total glycoproteins (including the acid hydrolases) by affinity chromatography (Con A-Sepharose), exhaustive dialysis against distilled water (several changes) and final concentration by ultrafiltration (Sartorius Membranfilter, Göttingen). The isolation of the hydrolases was followed by measuring enzyme activities with chromogenic substrates. (For details see ref. [13]).

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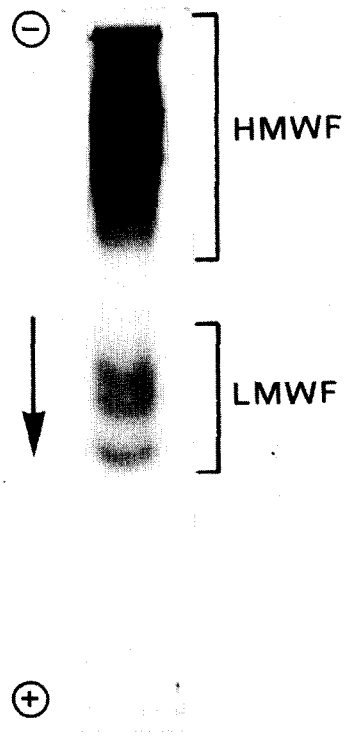

Fig.1. Fractions obtained after semipreparative polyacrylamide gel electrophoresis of the lysosol from secondary lysosomes. Electrophoresis was performed as in [18], except that a 15% gel was used. Proteins were stained with Coomassie Brilliant Blue R-250. (For details of method see [13]). HMWF, high mol. wt. fraction; LMWF, low mol. wt. fraction.

Highly purified sulphatase A was prepared according to Stinshoff from human liver [19].

2.4. *Substrates*

Galactosylceramide and cerebroside sulphate were prepared from bovine brain and labelled with tritium in the ceramide moiety as previously described [20,21]. Glucosylceramide and trihexosylceramide (GalNAc-β-1,4-Gal-1,4-Glc-1,1-(2-*N*-acyl)-sphingosine, the sialic acid-free residue of the ganglioside G_{M2}), also labelled with tritium in the ceramide moiety were gifts of Dr K. Harzer (Institut für Hirnforschung, Universität Tübingen) and Dr K. Sandhoff (Max-Planck-Institut für Psychiatrie, München), respectively. Sphingomyelin was labelled with ^{14}C in the choline moiety and was obtained from NEN Chemicals GmbH (Dreieichenhain, Germany).

2.5. *Assay procedures*

The assay of the enzymic hydrolysis of sphingolipids was performed according to described procedures [20–24]. The incubation mixtures (100 μl) contained, in addition to the lipid substrate, the following buffers: 20 μmol sodium acetate, pH 4.8 (for cerebroside sulphatase), 20 μmol sodium acetate, pH 4.5 (for cerebroside galactosidase and cerebroside glucosidase), 12.5 μmol sodium citrate, pH 4.0 (for β-*N*-acetylgalactosaminidase), 20 μmol sodium acetate, pH 5.0 (for sphingomyelinase) and appropriate amounts of enzyme and activator protein. The amounts of the appropriate substrate used in the incubation mixtures were 2 nmol and 20 nmol respectively in the cases of qualitative and quantitative determination of enzymic hydrolysis.

In all cases the lipid substrate was dispersed in the respective buffer by sonication (2 × 10 sec, 100 W; Branson Sonic Power Comp., Danbury, Conn.) after evaporation to dryness from organic solvents (benzene/ethanol 2:1, v:v). After incubation for various times (see Results), the mixtures were analyzed by radio thin-layer chromatography [25]. The areas representing substrate and product were cut out from Silica gel-G-plastic plates, and the radioactivity was measured in a liquid scintillation analyzer (Mark II; Nuclear Chicago, Des Plaines, Ill., USA) [26]. In the case of sphingomyelinase, substrate and reaction product were partitioned according to Folch [27] and the two phases washed twice with theoretical phases [27]. After evaporating to dryness the radioactivity of the lower and upper phases together with their washings was determined as described above.

3. Results

When incubated under physiological conditions without the addition of detergents, unfractionated disrupted secondary lysosomes hydrolyze sphingolipids, e.g., cerebroside sulphate, galactosylceramide, glucosylceramide, trihexosylceramide and sphingomelin (see fig.2).

As published in detail elsewhere [13], a high mol. wt. component from the soluble part of the lysosomes (lysosol) (cf. fig.1) could be isolated, which contained the acid hydrolases as detected by means of chromogenic substrates. These lysosomal enzymes, however,

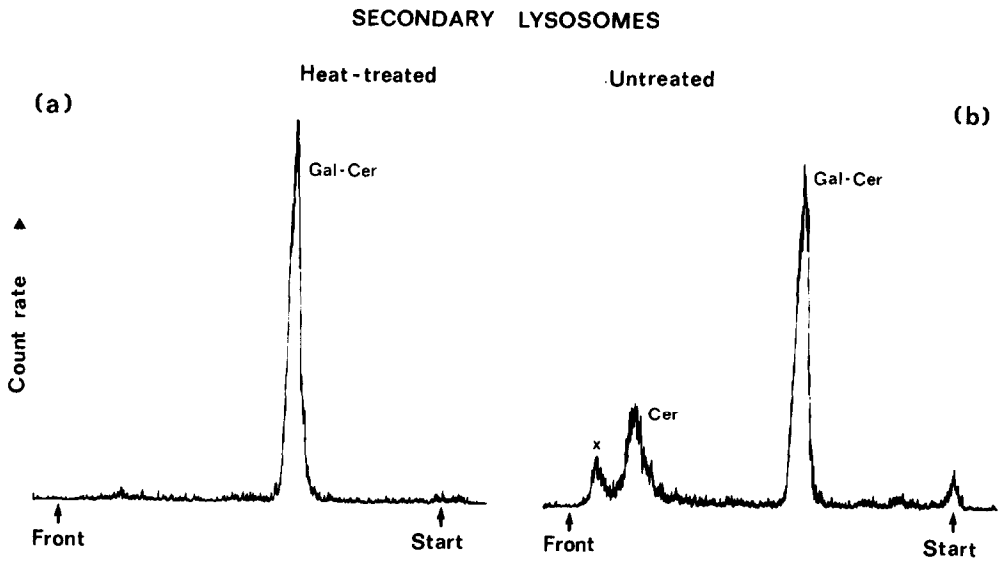

Fig. 2. Hydrolysis of galactosylceramide (^{3}H-labelled in the ceramide moiety) by unfractionated secondary (iron-loaded) lysosomes. Radio thin-layer chromatograms of the reaction product obtained. For further details see text. (a) Heat-treated organelles. (b) Untreated organelles. Gal, galactose; Cer, ceramide (*N*-acylsphingosine); Gal-Cer, galactosylceramide; x, unknown product (presumably free fatty acids).

showed no or only a slight activity towards their physiological sphingolipid substrates, unless detergents, e.g., taurodeoxycholate, were used as activators. The sphingolipid-hydrolyzing activity, however, could be restored, at least in part, by the addition of a low mol. wt., heat stable, protein fraction, exhibiting no enzymic activity, which was likewise isolated from the soluble part (lysosol) of secondary lysosomes (cf. fig.1).

This low mol. wt. activator fraction strongly stimulated sphingolipid hydrolysis when incubated with a highly purified enzyme, e.g. human sulphatase A using cerebroside sulphate as substrate. Similar results were obtained with a number of other sphingolipid hydrolases contained in the high mol. wt. fraction when measured in the absence or presence of the low mol. wt. fraction (fig.3, table 1). The extent of activation, however, was somewhat less compared to the highly purified sulphatase A. Surprisingly, no cerebroside sulphatase activity was obtained with the lysosomal enzyme fraction (apparently due to the fact that only approx. 10% of the total sulphatase present in rat liver lysosomes accounts for sulphatase A; unpubiished results).

The ratio of the activating effect of the low mol. wt. protein on the different sphingolipid hydrolases varied with the preparation obtained. One activator protein preparation was almost as potent as the highly purified activator of cerebroside sulphatase recently described [7], when using purified human sulphatase A as enzyme and cerebroside sulphate as substrate.

4. Discussion

The following observations suggest that an enzymically inactive heat stable low mol. wt. protein fraction, acts in the lysosomes as a physiological activator of the sphingolipid hydrolases: (a) Unfractionated, disrupted lysosomes hydrolyze cerebroside sulphate, galactosylceramide, glucosylceramide, trihexosylceramide and sphingomyelin (cf. fig.2; also [28,29]). (b) Acid sphingolipid hydrolases contained in a high mol. wt. protein fraction, obtained after semipreparative separation by gel electrophoresis from secondary lysosomes (fig.1) show no or only slight hydrolase activity towards their natural sphingolipid substrates,

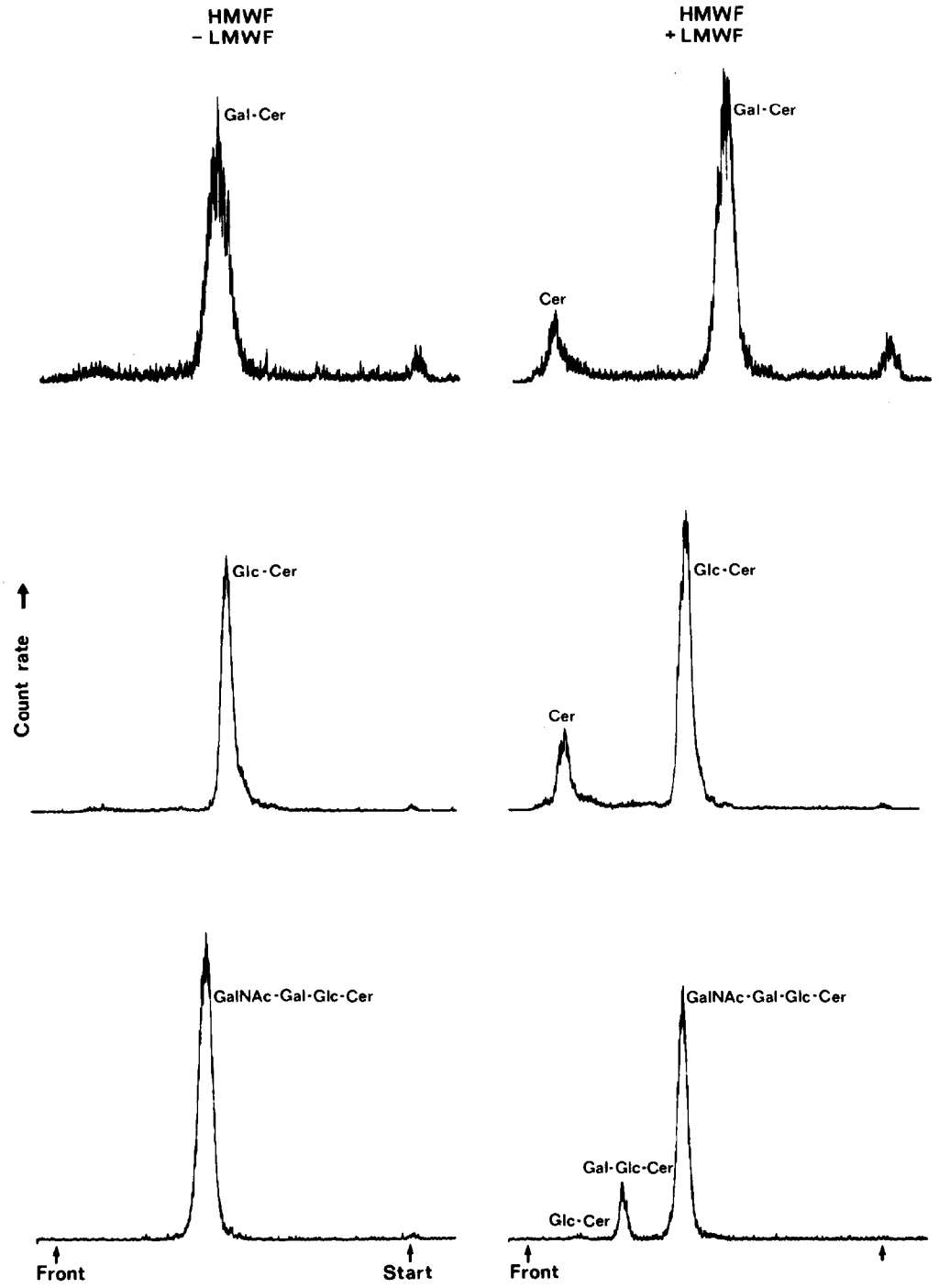

Fig.3. Scans of thin-layer radiochromatograms showing the result of the action of the low mol. wt. fraction (LMWF) on the enzymic hydrolysis of various sphingolipids by the high mol. wt. fraction (HMWF), both isolated from secondary lysosomes. For details see text. Gal, galactose; Glc, glucose, GalNAc, *N*-acetylgalactosamine; Cer, ceramide (*N*-acylsphingosine); Gal-Cer, galactosylceramide; Glc-Cer, glucosylceramide; GalNAc-Gal-Glc-Cer, trihexosylceramide (the sialic acid-free residue of the ganglioside G_{M2}).

Table 1
Sphingolipid hydrolase activities of the high mol. wt. fraction (HMWF), measured with physiological substrates in the absence and presence of the low mol. wt. fraction (LMWF)

Sphingolipid hydrolases (HMWF)	Activity towards chromogenic substrates (μmoles/min/mg protein)	Sphingolipid substrates (20 nmol/incubation mixture)	Sphingolipid hydrolysis (nmoles product formed/h)		Incubation time (h)
			− LMWF (control)	+ LMWF	
Sulphatase A (highly purified from human liver)	30.3	Cerebroside sulphate	0.028	1.210	2
Sulphatase A + B	1.61	Cerebroside sulphate	–[b]	–[b]	20
Cerebroside galactosidase	0.08	Galactosylceramide	–[b]	0.197	10
Cerebroside glucosidase	0.13	Glucosylceramide	0.119	0.297	10
β-*N*-Acetyl-galactosaminidase	0.75	Trihexosylceramide	0.018	0.238	5
Sphingomyelinase	–[a]	Sphingomyelin	0.022	0.538	2

Determinations were performed as described under Materials and methods with 4.5 μg of human sulphatase A or 200 μg of the HMWF and 170 μg of the LMWF. The values represent means of two determinations. Activities towards chromogenic substrates (*p*-nitrophenyl compounds) were measured according to [20,21].
[a] No chromogenic substrate available.
[b] Not detectable.

similar to highly purified lysosomal sulphatase A (fig.3, table 1). (c) The sphingolipid cleaving activity of the respective lysosomal enzymes could be restored, at least in part, by the addition of the lysosomal low molecular weight protein fraction.

On a weight-to-weight basis, the activating capacity of this protein component was almost in the same order of magnitude as the highly purified activator of cerebroside sulphatase prepared by a completely different procedure from human liver [7], when measured with pure human sulphatase A and cerebroside sulphate as substrate (table 1). The lower activation of sphingolipid hydrolases separated from lysosomes, may be attributed to the low enzyme activities in our preparation, as well as to contamination with other (enzyme) proteins which might lead to an unspecific inhibition of the enzymic reactions [30].

It cannot be decided at present whether a universal activator protein stimulates all the sphingolipid hydrolases, or if different molecular species with very similar properties activate the different hydrolytic reactions (or groups of sequential steps). Since the activator protein obtained seems to be heterogeneous, as revealed by the gel electrophoretic behaviour (fig.1), the latter possibility cannot be excluded.

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References

[1] Bowen, D. M. and Radin, N. S. (1968) Biochim. Biophys. Acta 152, 587–598; and Bowen, D. M. and Radin, N. S. (1968) Biochim. Biophys. Acta 152, 599–610.

[2] Jerfy, A. and Roy, A. B. (1973) Biochim. Biophys. Acta 293, 178–190; and Stinshoff, K. and Jatzkewitz, H. (1975) Biochim. Biophys. Acta 377, 126–38.
[3] Lowenstein, J. M. (ed.) (1969) Methods in Enzymology, 14, Academic Press, New York.
[4] Gatt, S. and Barenholz, Y. (1973) Ann. Rev. Biochem. 42, 61–90.
[5] Mehl, E. and Jatzkewitz, H. (1964) Hoppe-Seyler's Z. Physiol. Chem. 339, 260–76.
[6] Mehl, E. and Jatzkewitz, H. (1968) Biochim. Biophys. Acta 151, 619–627.
[7] Fischer, G. and Jatzkewitz, H. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 605–13.
[8] Ho, M. W. and O'Brien, J. S. (1971) Proc. Nat. Acad. Sci. (USA) 68, 2810–13.
[9] Ho, M. W., O'Brien, J. S., Radin, N. S. and Erickson, J. S. (1973) Biochem. J. 131, 173–76.
[10] Li, Y.-T., Mazzotta, M. Y., Wan, C.-C., Orth, R. and Li, S.-C. (1973) J. Biol. Chem. 248, 7512–15.
[11] Li, S.-C., Wan, C.-C., Mazzotta, M. Y. and Li, Y.-T. (1974) Carbohyd. Res. 34, 189–93.
[12] Li, S.-C. and Li, Y.-T. (1976) J. Biol. Chem. 251, 1159–1163.
[13] Mraz, W., Fischer, G. and Jatzkewitz, H., Hoppe-Seyler's Z. Physiol. Chem., in the press.
[14] Trouet, A. (1974) Methods Enzymol. 31, 323–29.
[15] Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. and de Duve, C. (1968) J. Cell Biol. 37, 482–513.
[16] Arborgh, B., Ericsson, J. L. E. and Glaumann, H. (1973) FEBS Lett. 32, 190–94.
[17] Glaumann, H., Jansson, H., Arborgh, B. and Ericsson, J. L. E. (1975) J. Cell Biol. 67, 887–94.
[18] Williams, D. E. and Reisfeld, R. A. (1964) Ann. N. Y. Acad. Sci. 121, 373–81.
[19] Stinshoff, K. (1972) Biochim. Biophys. Acta 276, 475–90.
[20] Sandhoff, K., Harzer, K., Wässle, W. and Jatzkewitz, H. (1971) J. Neurochem. 18, 2469–89.
[21] Mraz, W. and Jatzkewitz, H. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 33–44.
[22] Bowen, D. M. and Radin, N. S. (1969) J. Neurochem. 16, 501–11.
[23] Brady, R. O., Kanfer, J. and Shapiro, D. (1965) J. Biol. Chem. 240, 39–43.
[24] Kanfer, J. N., Young, O. M., Shapiro, D. and Brady, R. O. (1966) J. Biol. Chem. 241, 1081–84.
[25] Harzer, K., Stinshoff, K., Mraz, W. and Jatzkewitz, H. (1973) J. Neurochem. 20, 279–87.
[26] Sandhoff, K., unpublished procedure.
[27] Folch, J. Lees, M. and Sloane Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509.
[28] Mellors, A. and Tappel, A. L. (1967) J. Lipid Res. 8, 479–85.
[29] Weinreb, N. J., Brady, R. D. and Tappel, A. L. (1968) Biochim. Biophys. Acta 159, 141–46.
[30] Stinshoff, K. (1974) Dissertat. Univ. München.